Clinical Cancer Investigation Journal-ISSN: 2278-0513

Investigating the Effects of Metformin on Heart Tissue and Related Enzymes in Non-Diabetic Male Rats

Abstract

Metformin is a hypoglycaemic agent and an oral antidiabetic drug from the biguanide category and is the first line of treatment for type 2 diabetes, especially in obese people with normal kidney function. Metformin effectively improves insulin sensitivity and endothelial function, improving cardiovascular conditions in people with diabetes and controlling body weight. In the current study, the effect of metformin has been investigated on the cardiac tissue of its enzymes in adult non-diabetic male rats. In the beginning, a metformin solution was prepared. After determining the appropriate dose of metformin, intraperitoneal injection was administered to 30 rats within 30 days with doses of 15 mg/kg.b.w (first group), 20 mg/kg.b.w (second group), mg/kg.b.w 25 (third group), control group (no injection) and sham (distilled water injection). The data was measured with $SPSS_{22}$, the ANOVA test, and Duncan's test with a significance level of ($P \leq 0.05$). In the macroscopic examinations, a significant weight decrease was observed in all three injection doses ($P \leq 0.001$). In the case of heart weight, the reduction was not significant. A significant increase was observed in measuring the diameter of the left and right ventricles of the heart ($P \leq 0.05$). In the examination of enzyme parameters, there were no significant changes in cardiac CPK enzyme; a significant decrease was observed in cardiac LDH and CKMB enzymes ($P \leq 0.05$). In the microscopic examination of the heart tissue, changes in the arrangement of cells and nuclei, an increase in the number of nuclei and interstitial space, and a decrease in the number of blood capillaries were observed. Generally, it can be concluded that the consumption of different doses of metformin in rats has the same negative effect on their bodies because it causes a destructive effect on the heart tissue and also causes a decrease in the number of enzymes in this tissue below the normal level in the body. Consequently, its use should be done under the supervision of a doctor and consciously.

Keywords: *Metformin, Rat, Heart, Cardiac enzymes.*

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Introduction

Metformin is an old drug and an oral hypoglycemic agent used to treat patients with non-insulin-dependent diabetes (type II diabetes), which is mainly used to reduce blood sugar in type II diabetes. This drug is a biguanide class of antidiabetic agents (1). Metformin does not cause hypoglycemia (severe blood sugar drop) and reduces gluconeogenesis, which leads to a decrease in hepatic glucose output (2). Other pharmacological activities of metformin include increasing glucose oxidation and its storage in the form of glycogen and fat, inhibiting fatty acid oxidation and reducing glucose absorption (3). Several studies have shown that metformin exerts beneficial metabolic effects by activating AMP protein kinase (AMPK) (4 & 5).

The heart is a muscular organ in humans and other animals that circulates blood through blood vessels in the circulatory system. The blood delivers oxygen and necessary nutrients and removes waste products from metabolism (6). The enzyme that is usually measured to confirm the presence of damage in the heart muscle is creatine kinase (CK). Different types of CK are found in cardiac muscles and skeletal muscles. The enzyme that is the most precise indicator of heart damage is a form of CK known as CK-MB. The level of CK-MB found in the blood increases about 6 hours after the onset of a heart attack and reaches its peak after about 18 hours (7, 8). CPK is an intracellular enzyme concentration that is high in skeletal muscle, myocardium, and brain, and damage to each of these tissues causes an increase in serum CPK level (9). CK may help diagnose and treat inflammatory myositis, muscular dystrophy, myocardial diseases, and rhabdomyolysis (9, 10 & 11).

For a more specific examination of myocardial electrophoresis muscle lesions, creatine kinase MB measurement is done to determine the isoenzymes of creatine phosphokinase. These isoenzymes (MB), CPK2 (CPK-CPK-BB) (CPK1), and (CPK-MM) (CPK3) increase three to six hours after infarction. If there is no new damage to the myocardium, the CPK-MB level reaches its peak 12 to 14 hours later and returns to normal 12 to 48 hours after the infarction (7 & 8).

LDH, also known as LD or lactate dehydrogenase enzyme, is an enzyme that exists in almost all tissues of the body, particularly the heart, liver, red blood cells, kidneys, skeletal muscles, brain, and lungs. Usually, in healthy people and in the absence of tissue and cell damage, only a small amount of it is present in the blood. As a result of cell damage, LDH is released from inside the cells and enters the bloodstream, where its level increases in the blood. Due to this, LDH can be a good indicator for evaluating tissue and cellular damage (12 & 13).

Shafizadeh et al. (2012) examined the effects of metformin on glucose, lipid profiles, and serum oxidative stress in alloxan-induced diabetic rats. The results revealed that in diabetic rats, metformin significantly reduced glucose, triglycerides,

cholesterol, LDL, and VLDL and caused an increase in serum HDL (14). Sun et al. (2017) conducted research entitled "Metformin improves cardiac function in rats with heart failure after myocardial infarction by regulating mitochondrial energy metabolism." The results disclosed that metformin improved the systolic function of rats and reduced the apoptosis of myocardial cells after myocardial infarction (15).

Nasrallahi et al. (2013) conducted studies on "the effect of honey and metformin in improving the damage caused by diabetes in the rat testis and its relationship with hormonal changes." Biochemical analysis indicated that diabetes significantly decreased serum testosterone levels, LH levels, and FSH in the serum. Likewise, the light microscopic analysis indicated a significant decrease in the diameter of the spermatogenic tubules and the thickness of the epithelium of the diabetic group compared to the control group and the group treated with metformin and honey. The concurrent administration of honey and metformin could regulate the serum levels of testosterone, LH, and FSH at an acceptable level. Histopathological analyses showed the improvement of atrophy of the spermatogenic tubules and an increase in the thickness of the epithelium, the diameter of the spermatogenic tubules and the spermatogenesis index following the treatment (16). Lyons et al. (2013) studied the effect of gender on the functional and metabolic reactions of the heart in diabetes treatment. The results showed that no significant therapeutic effect was found for the metabolism of the myocardial layer because, to some extent, men and women had different reactions to a certain treatment (17).

The current research aimed to investigate the effects of metformin on the heart tissue of the enzymes creatine kinase MB, creatine phosphokinase and lactate of Cardiac dehydrogenase in non-diabetic male rats.

Method

This study examined the effects of metformin on heart tissue and creatine kinase MB, creatine and phosphokinase, and cardiac lactate dehydrogenase enzymes in adult non-diabetic male rats. The tissue similarities to human tissue and the ease of their maintenance and breeding are among the reasons for choosing this type of animal. Adult non-diabetic male laboratory rats weighing 300 to 330 grams and with an age range of 2.5 to 3 months were purchased from Razi Serum and Vaccination Institute in Karaj. They were kept in the animal room of Islamic Azad University, Karaj branch, under controlled conditions in terms of temperature and humidity. Via an automatic electric timer, a period of 12 hours of light and 12 hours of darkness was established. The shelves were washed every 3 days to prevent contamination, and the straw was changed. The temperature of the room was set at 22° C, and the humidity of the room was set to normal with a humidifier. The statistical population includes five groups: 1. the control group that does not receive injections; 2. a sham group, which received distilled water injections for 30 days before being killed the day after the last injection; 3. in experimental group 1, the samples were injected with metformin in the amount of 15 mg/kg for 30 days and were killed the day after the last day of injection; 4. in experimental group 2, the samples were injected with metformin in the amount of 20 mg/kg for 30 days and were killed the day after the last day of injection; 5. in experimental group 3, the samples were injected with metformin at 25 mg/kg for 30 days and were killed the day after the last day of injection; each group included 6 rats.

To determine the $LD50$, metformin doses of 15mg/kg, mg 20/kg, and 25 mg/kg were used. The 500 mg metformin tablets were purchased from a pharmacy. The tablet was powdered, then the amounts of 0.4 mg of metformin powder for a dose of 15 mg/kg, the amount of 0.6 mg of metformin powder for a dose of 20 mg/kg, and the amount of 0.7 mg of metformin powder for a dose of 25 mg/kg were dissolved and diluted with distilled water using a new digital weight scale with 0.2 cc. Next, the solution was passed through the Whatman filter paper to obtain the filtered solution. In all stages of metformin injection, the injection was done intraperitoneally in the groin area of the thigh with the help of a 1 mL insulin syringe. One day after the last injection of the animals, the rats were anesthetized with chloroform after weighing, and blood was taken from the heart by inserting a syringe into the chest and heart area. To prevent hemolysis, by removing the syringe head, the blood was slowly poured into 2 mL microtubes and kept for one hour at 32 degrees to clot and prepare the serum. Then the microtubes were centrifuged in a special microtube centrifuge for 5 minutes at 3000 rpm, the clot was settled, and the blood serum was separated by a sampler and stored in 1.5 mL microtubes to be stored in a freezer at -18°C for cardiac enzyme measurement. Then, at a suitable time, all serums were taken out of the freezing state by staying at room temperature for half an hour, and the concentration of each enzyme was measured by an auto analyzer and lactate dehydrogenase, creatine kinase MB and cardiac creatine phosphokinase enzyme kits.

For the microscopic study, the hearts of the rats were removed from the bodies after being killed, and then the weight of the hearts was measured using a digital scale in the control, sham, and experimental groups. At this stage, the heart was placed in 10% formalin for 48 hours to be fixed for tissue procedures. After sampling, the samples were painted. For a more detailed examination of the heart tissue, the prepared samples were carefully examined by stereomicroscope, and the thickness and diameter of the left and right ventricles of the heart were measured with the help of calipers. The general arrangement of cells and the shape and number of cell nuclei were also

evaluated by an optical microscope. Each experimental sample was compared with the control and sham samples, and finally, photoFigures of the desired sections were taken. SPSS software, ANOVA, and Duncan's method were used to analyze the collected data. The significance limit of the tests was considered to be ≤ 0.05. Lastly, the diagrams were drawn by SPSS software.

Research results

Body weight

In the investigations carried out on the difference in weight before the first. After the last injection, it was determined that in the control and sham groups and all 3 experimental groups with injection doses of 25, 20, and 15 mg/kg, the secondary weight of the animals had increased compared to their initial weight, but by examining the average secondary weight minus the initial weight in the groups, a significant decrease ($P<0.001$) was observed in the weighing of the experimental groups compared to the control and sham groups. Likewise, no significant difference was observed between the experimental groups, and this means the control of weight gain in the experimental groups after taking metformin (Table 1)

Table 1. Weight difference before the first and after the last injection in terms of (gr)

Studied groups	**SD**	**Mean**	**Significance compared to the control group, sham and between groups**
Control	±13.876	61.66	
Sham	±20.643	61.33	
Experimental dose 15 mg/kg.b.w (T1)	±25.277	46.66	aa $P < 0.001$
Experimental dose 20 mg/kg.b.w (T2)	±11.057	29.66	aa $P < 0.001$
Experimental dose 25 mg/kg.b.w (T3)	±22.713	29.50	aa $P < 0.001$

aa = significance of P <0.001 compared to the control and sham groups

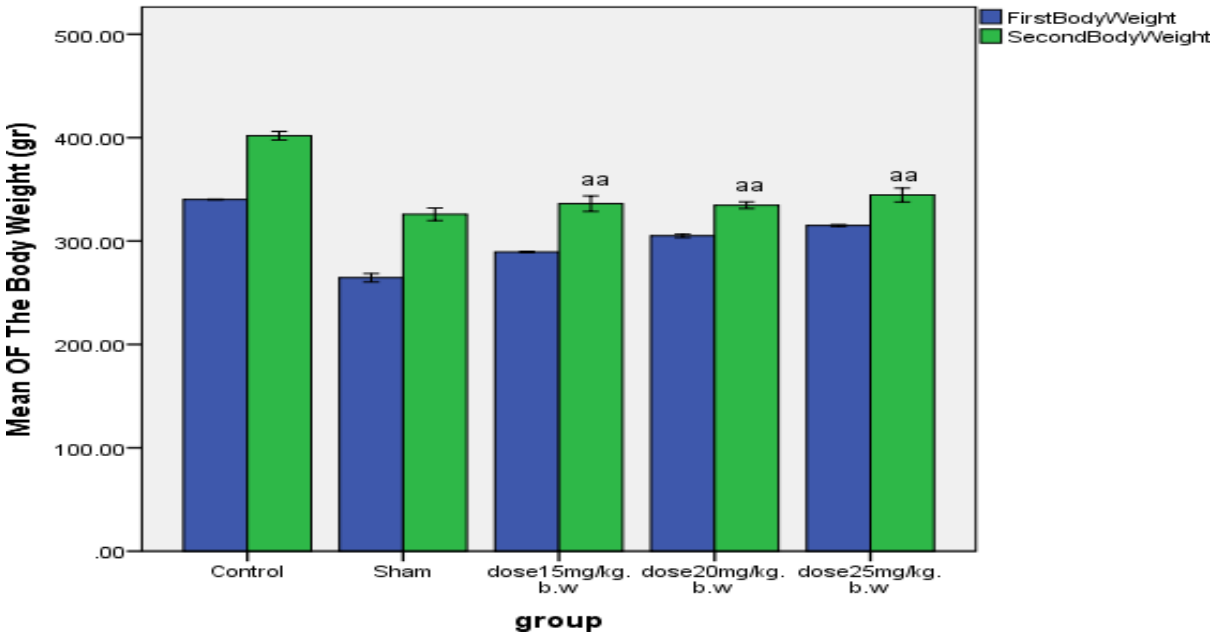

Diagram 1: Comparison of body weight of rats in the control, sham and experimental samples
Significance of <0.001= aa

Heart weight

In the studies conducted on heart weight, in experimental groups 1, 2, and 3 with injection doses of 25, 20, and 15 mg/kg, we witnessed a decrease compared to the control and sham groups, but these changes were not significant (Table 2).

Table 2: Heart weight in different doses in (gr)

Studied groups	SD	Mean	Significance compared to the control group, sham and between groups
Control	±0.045	1.600	
Sham	±0.026	1.574	
Experimental dose 15 mg/kg.b.w (T1)	±0.133	1.495	It is not significant
Experimental dose 20 mg/kg.b.w (T2)	±0.154	1.438	It is not significant
Experimental dose 25 mg/kg.b.w (T3)	±0.191	1.569	It is not significant

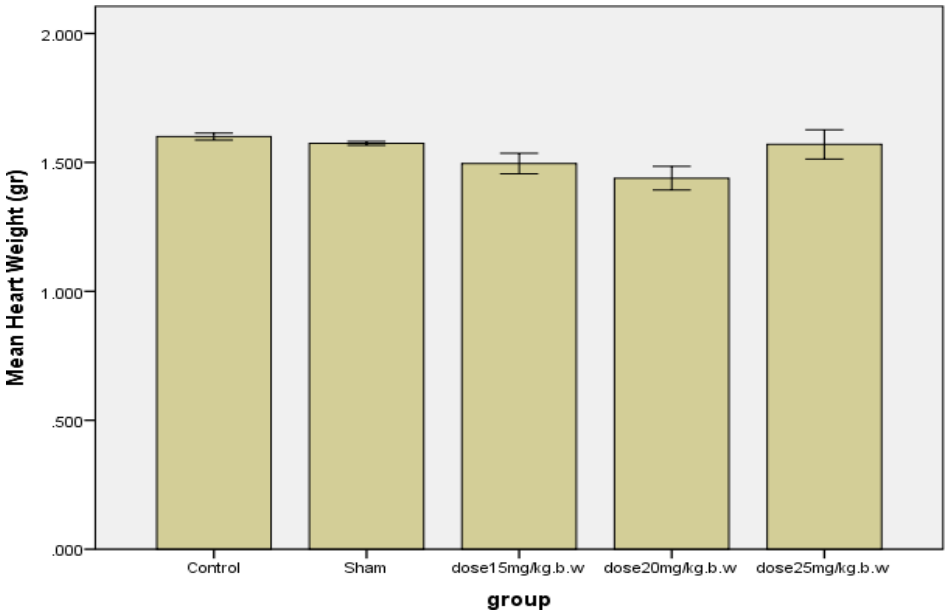

Diagram 2: Comparison of heart weight of rats in the control, sham and experimental samples

Results of the macroscopic examination of the diameter of the left ventricle

In the investigations, it was found that the diameter of the left ventricle in the experimental groups 1 and 3 with injection doses of 25 and 15 mg/kg compared to the control and sham groups was significantly increased by (P<0.001) and in experimental group 2, it was increased significantly (P<0.05) with an injection dose of 20 mg/kg (Table 3).

Table 3: Diameter of the left ventricle of the heart in different doses in (mm)

Studied groups	SD	Mean	Significance compared to the control group, sham and between groups
Control	±0.795	2.805	
Sham	±0.181	2.988	
Experimental dose 15 mg/kg.b.w (T1)	±0.970	5.091	aa $P < 0.001$
Experimental dose 20 mg/kg.b.w (T2)	±0.275	4.156	a $P < 0.05$
Experimental dose 25 mg/kg.b.w (T3)	±0.379	4.676	aa $P < 0.001$

aa = significance ($P < 0.001$) compared to the control and sham groups

a = significance ($P < 0.05$) compared to the control and sham groups

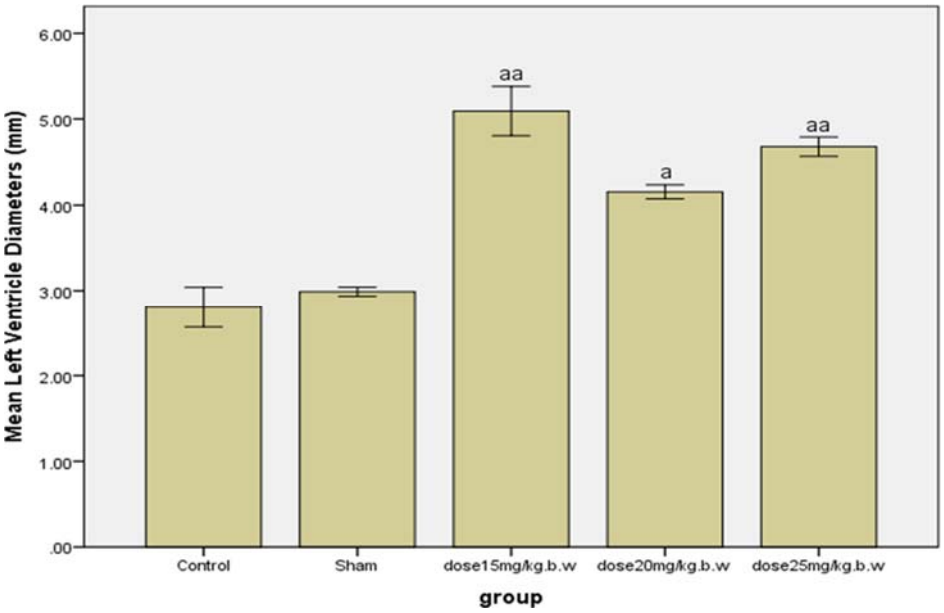

Diagram 3: Comparison of the left ventricular diameter of rats in the control, sham and experimental samples
a = significance of P< 0.05
aa = significance of *P* <0.001

Results of the macroscopic examination of the diameter of the right ventricle of the heart

In the studies, it was found that the diameter of the right ventricle in experimental groups 1 and 3 with injection doses of 25 and 15 mg/kg was significantly increased compared to the control and sham groups, and in experimental group 2 with an injection dose of 20 mg/kg increased, but this increase was not significant (Table 4).

Table 4: Diameter of the right ventricle of the heart in different doses in (mm)

Studied groups	**SD**	**Mean**	**Significance compared to the control group, sham and between groups**
Control	±0.736	1.895	
Sham	±0.447	1.843	
Experimental dose 15 mg/kg.b.w (T1)	±0.325	2.696	a $P < 0.05$
Experimental dose 20 mg/kg.b.w (T2)	±0.483	2.628	It is not significant
Experimental dose 25 mg/kg.b.w (T3)	±0.401	3.036	a $P < 0.05$

a = significance ($P < 0.05$) compared to the control and sham groups

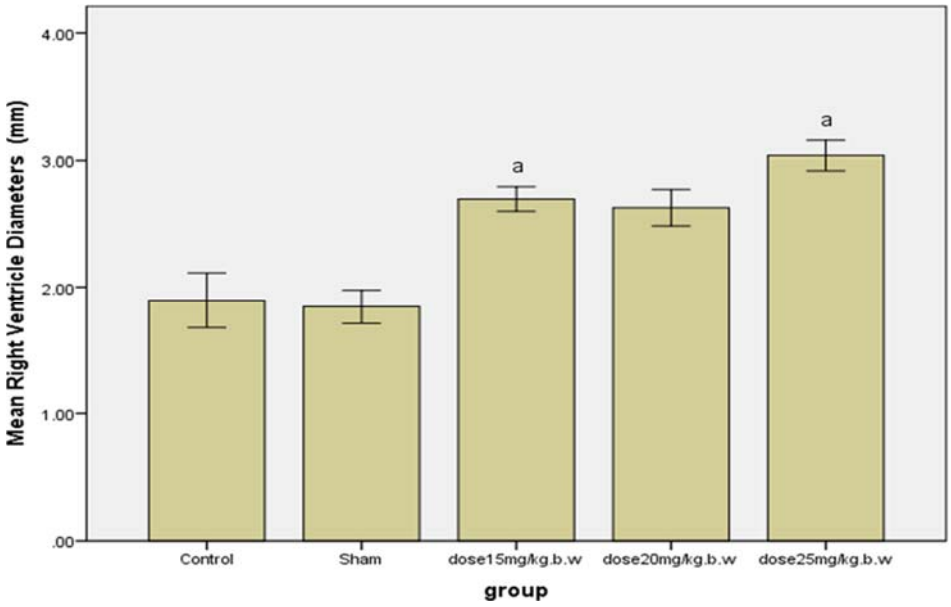

Diagram 4: Comparison of the diameter of the right ventricle of rats in the control, sham and experimental samples
a = significance of P< 0.05

Results of cardiac creatine phosphokinase (CPK)

In the investigations, it was found that the amount of this enzyme in experimental groups 1, 2, and 3 with injection doses of 25, 20, and 15 mg/kg decreased compared to the control and sham groups, but none of the changes were significant (Table 5.)

Table 5: Cardiac creatine phosphokinase (U/L) (CPK) enzyme results

Studied groups	**SD**	**Mean**	**Significance compared to the control group, sham and between groups**
Control	±412	1022	
Sham	±440	1007	
Experimental dose 15 mg/kg.b.w (T1)	±417	416	It is not significant
Experimental dose 20 mg/kg.b.w (T2)	±259	475	It is not significant
Experimental dose 25 mg/kg.b.w (T3)	±548	726	It is not significant

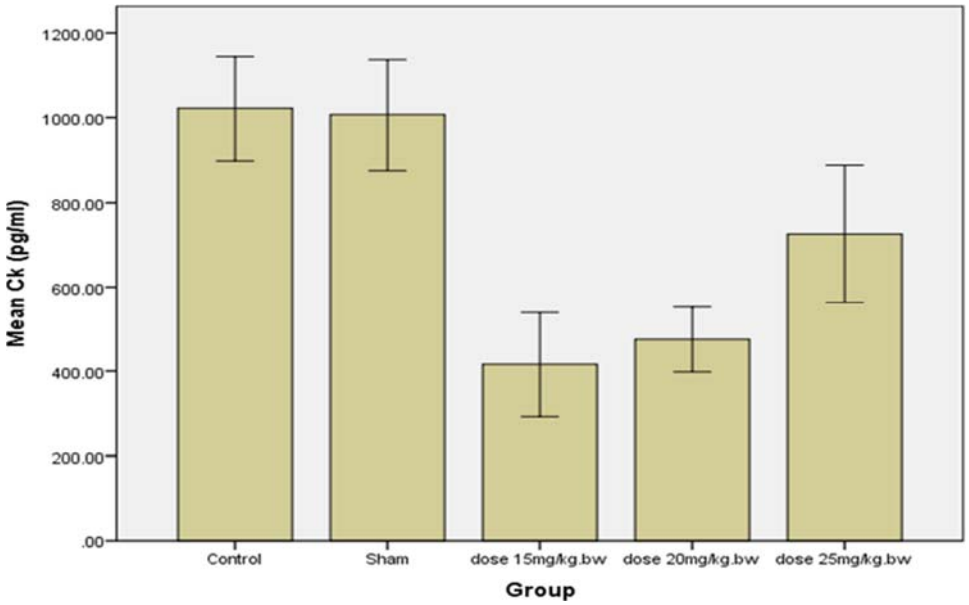

Diagram 5: Comparison of rat CPK enzyme in control, sham and experimental samples

Results of cardiac creatine kinase MB (CKMB)

With the investigations, it was found that the amount of this enzyme in the experimental groups 1, 2 and 3 with injection doses of 25, 20, and 15 mg/kg compared to the control and sham groups was significantly reduced (P<0.05) (Table 6).

Table 6: Cardiac Creatine MB Enzyme Results (U/L) (CKMB)

Studied groups	**SD**	**Mean**	**Significance compared to the control group, sham and between groups**
Control	±358	1097	
Sham	±405	1060	
Experimental dose 15 mg/kg.b.w (T1)	±237	404	a $P < 0.05$
Experimental dose 20 mg/kg.b.w (T2)	±189	357	a $P < 0.05$
Experimental dose 25 mg/kg.b.w (T3)	±351	410	a $P < 0.05$

a=Significance ($P < 0.05$) compared to the control and sham groups

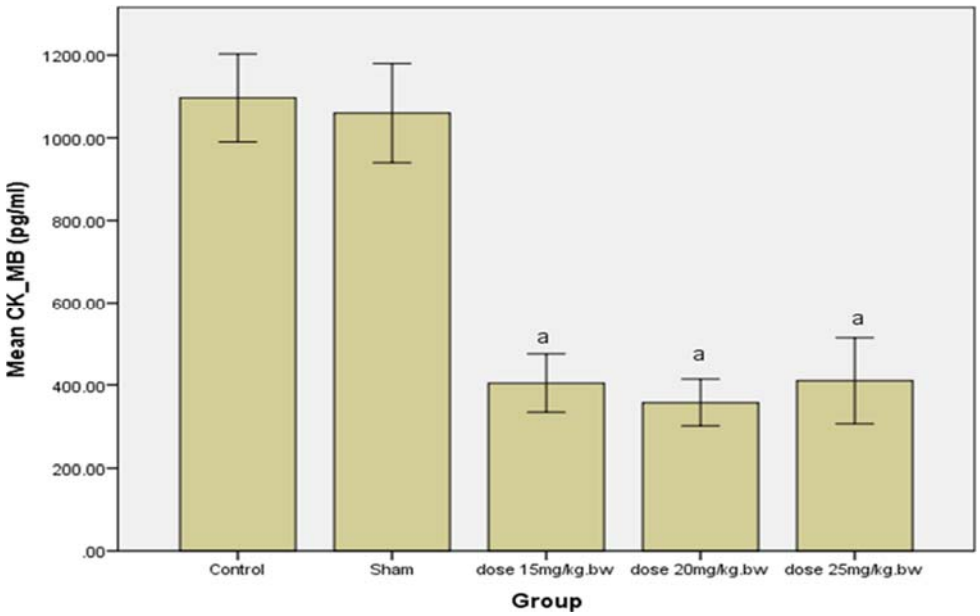

Diagram 6: Comparison of CKMB enzyme of rats in the control, sham and experimental samples
a = with significance of (P< 0.05)

Results of cardiac lactate dehydrogenase (LDH)

Via the investigations, it was found that the amount of this enzyme in the experimental groups 1, 2 and 3 with injection doses of 25, 20, and 15 mg/kg compared to the control group had been significantly reduced (P<0.05) (Table 7).

Table 7: Results of cardiac lactate dehydrogenase enzyme (U/L) (LDH)

Studied groups	**SD**	**Mean**	**Significance compared to the control group, sham and between groups**
Control	±470	1455	
Sham	±638	1362	
Experimental dose 15 mg/kg.b.w (T1)	±358	406	a $P < 0.05$
Experimental dose 20 mg/kg.b.w (T2)	±268	383	a $P < 0.05$
Experimental dose 25 mg/kg.b.w (T3)	±280	430	a $P < 0.05$

a=Significance ($P < 0.05$) compared to the control and sham groups

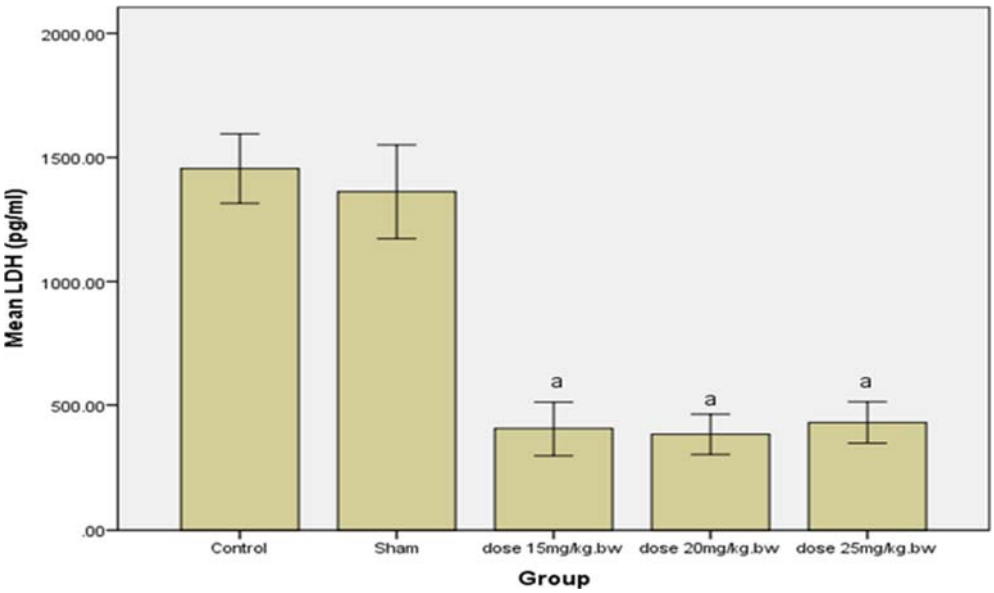

Diagram 7: Comparison of LDH enzyme of rats in the control, sham and experimental samples
a = with significance of (P< 0.05)

Discussion and conclusion

Metformin is a hypoglycaemic drug and an oral antidiabetic drug from the biguanide category and is the first line of treatment for type 2 diabetes, especially in obese people with normal kidney function. As well as the mentioned cases, extensive studies and research have shown that metformin has a promising and wide spectrum in the treatment of diseases such as polycystic ovary syndrome, gestational diabetes, fatty liver, lipodystrophy syndrome, breast cancer, lung cancer, prostate cancer, ovarian cancer, and pancreatic cancer. The current study investigated the effects of metformin on cardiac tissue and cardiac enzymes such as creatine phosphokinase, creatine kinase MB, and lactate dehydrogenase in adult non-diabetic male rats.

Lotfi et al. (2015), with an experiment on 48 NMIR rats, found that metformin led to a significant decrease in blood sugar, triglyceride cholesterol levels, and weight loss in the experimental groups compared to the control group, and due to the decrease in blood sugar, physical activity and mobility decreased in rats (18). Kayani Fard et al. (2011) found that the use of metformin in diabetic rats causes a decrease in body weight and testicular tissue changes compared to the control group. Consequently, the results of the current research are consistent with the results of previous researchers in that the injection of metformin drug in all three injection doses caused a significant decrease in the weight gain of rats, which is due to the fat-burning property of metformin and the reduction of appetite that it causes. Also, during the injection, we saw loose stools in the rats, all mentioned in the abovementioned studies.

branched cells with one or two oval nuclei in the center of the cell and clear interstitial space with many capillaries indicates the normal tissue of the heart.

Yin et al. (2011) found that metformin caused a smaller infarct size, reduced left ventricular dilatation, and maintained the percentage of blood ejected in each ventricular contraction in non-diabetic rats with MI heart failure (20). The research results show an increase in the diameter of the left and right ventricles in all three injected doses, which is contrary to Yin's results. The reason for this difference could be in the rats used because the rats studied by Yin had MI heart failure, but the rats in the current study were healthy. Xie et al. (2011) stated that long-term treatment with metformin significantly increased autophagy activity and preserved heart function in OVE26 diabetic rats but not in DN-AMPK diabetic rats (21). Cadeddu et al. (2015) specified that treatment with metformin alone or with exercise significantly increased long-term left ventricular systolic function at rest. These findings support the observation that the use of metformin alone or with exercise plays a vital role in dealing with the negative effects of insulin resistance on cardiovascular function (22). Sun et al. (2017) found that metformin improved the systolic function of rats suffering from heart failure after myocardial infarction and reduced the apoptosis of myocardial cells. Likewise, metformin has significantly improved the function of mitochondrial respiration, reduced the damage to the mitochondrial membrane potential, and improved the function of mitochondrial respiration (15). Lyons et al. (2013) found that metformin reduces fatty acid excretion in diabetic men, which is associated with increased plasma fatty acid levels, myocardial fatty acid utilization and oxidation, and decreased myocardial glucose utilization (17). Bai et al. (2013) found that metformin inhibits angiotensin II-induced by myofibroblast

differentiation by suppressing the production of reactive oxygen species through the inhibition of the PKC-NADPH oxidase pathway in adult rat cardiac fibroblasts.

The current study found that using metformin at three different injection doses of 15, 20, and 25 mg/kg in rats had the same effect on reducing heart weight as the control and sham groups. However, this weight loss was not significant, and there was a significant increase in the diameter of the left ventricle in all three injected doses and a significant increase in the diameter of the right ventricle in the injected doses of 15 and 25 mg/kg, and there was no significant increase in the 20th dose compared to the control and sham groups. Among the things that cause hypertrophy, we can mention the growth of cardiac muscle fibers, changes in the structure of the heart, the tendency of the heart to metabolize glucose, fibrosis of the cardiac muscle cells, and disorders in the function of the myocardium, which we have seen in the present study. Macroscopic changes have been accompanied by microscopic changes. These changes include changes in cell arrangement, changes in the shape of the nucleus, an increase in the number of nuclei, an increase in the interstitial space, and a decrease in the number of blood capillaries, which were visible in all three injected doses. What emerges from the comparison of the present study with previous studies is that the results are inconsistent. Metformin is prescribed for treating diabetes and in patients with diabetes and heart failure simultaneously. Metformin is a suitable drug, and because the rats in the current study were healthy, the use of metformin in them causes a destructive effect on heart tissue.

Diabetes increases the incidence of heart failure due to the increase in sugar and fat in the blood, which causes atherosclerosis of the coronary arteries. Hardening and thickening of the walls of blood vessels is a clear sign of heart sclerosis. Laboratory studies show that metformin improves cardiac muscle function by activating the AMP-activated protein (Kinase) signaling mechanism, which is completely independent of the anti-hyperglycemic effects of this drug. Taken together, studies show that metformin can act as a protective agent by strengthening the heart's function at the molecular level of heart disease, and even regardless of its effect in the treatment of diabetes, it can be used as a treatment for heart failure (20).

CPK enzyme is an intracellular enzyme that is abundant in skeletal muscle, myocardium and brain, and damage to any of these tissues raises serum CPK levels. The MB isoform signifies the myocardial origin of CPK. Most patients with polymyositis and dermatomyositis have high levels of CK. High levels of MB may be due to inflammatory damage to muscle or muscle regeneration. Lower levels A normal level of CK can indicate a poor prognosis, particularly in patients who develop dermatomyositis following malignancy. Those who have low muscle mass, severe dermatomyositis and polymyositis, liver diseases caused by alcohol and the first months of pregnancy, and rheumatoid arthritis are other factors that decrease this enzyme (7 & 24). The LDH enzyme is found in many body tissues, especially the heart, liver, red blood cells, blood, kidneys, skeletal muscles, brain, and lungs. Following time damage or cell disorder, cells containing LDH are lysed. Therefore, LDH enters the bloodstream and its levels are higher than normal. In cases such as myocardial infarction, lung diseases, liver diseases, skeletal muscle damage, all parenchymal diseases, testicular tumors, and diffuse injuries, we see an increase in the amount of this enzyme (25 and 18).

In this study, the CPK enzyme decreased in all three injection doses of 15, 20, and 25 mg/kg compared to the control and sham groups. Also, LDH and CKMB enzymes have significantly decreased in all three injection doses. Membrane lipid peroxidation causes the release of cytosolic enzymes such as LDH. LDH is an indicator of the toxicity of a chemical substance and cell lysis (26). Enzymes increase compared to the normal state due to the increase in tissue damage and their leakage into the serum, which is observed in cardiac patients, and the use of metformin in these people causes the reduction of cardiac enzymes and their recovery. Hence, the reduction of enzymes in healthy rats under the study and the microscopic changes we saw contradict the previous findings. By reviewing the studies on cardiac patients, metformin improves cardiac conditions by changing the phosphorylation status of AMP kinase and regulating the insulin signaling pathway in the myocardium. Among the reasons for the discrepancy between the findings of the present study and the previous studies, we can mention the change in the signaling and biochemical pathways in the heart tissue, which, of course, is beyond the scope of the present study and requires more studies.

Consequently, in the current research, the injection of all three doses of 15, 20, and 25 mg/kg of metformin in non-diabetic male rats had the same effect on reducing the weight of the rats, reducing the weight of the heart tissue, increasing the diameter of the left and right ventricles of the heart, and reducing the enzymes CKMB, LDH, and CPK. Because, according to the statistical analysis, in none of the mentioned cases, there was no difference between the groups in the experimental groups. Similarly, the same tissue changes were observed in the experimental groups compared to the control and sham groups in the microscopic examinations. So, the results of this study show the harmful effects of metformin on the heart tissue and its enzymes in healthy people. Future studies are required to gain a precise understanding of the site and mechanism or the molecular and cellular mechanisms effective in the pharmacological action of metformin.

Acknowledgments
None.
Conflict of interest
None.
Financial support
None.
Ethics statement
None

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